TÜBİTAK

Turkish Journal of Veterinary and Animal Sciences

http://journals.tubitak.gov.tr/veterinary/

Research Article

Turk J Vet Anim Sci
(2020) 44: 227-234
© TÜBİTAK
doi:10.3906/vet-1911-20

Expression profiles of lincRNA and mRNA related to milk yield and milk composition traits in the milk-derived exosomes of Holstein and Doğu Anadolu Kırmızısı cows

Selçuk ÖZDEMİR[1,*], Ömer ELTAS[2], Muhammed Hüdai ÇULHA[1]

[1]Department of Genetics, Faculty of Veterinary Medicine, Atatürk University, Erzurum, Turkey
[2]Department of Biometry, Faculty of Veterinary Medicine, Atatürk University, Erzurum, Turkey

Received: 06.11.2019 • **Accepted/Published Online:** 22.02.2020 • **Final Version:** 06.04.2020

Abstract: This study aimed to demonstrate the expression profiles of lincRNAs and mRNAs affecting milk yield and composition traits in the milk-derived exosomes of Holstein and Doğu Anadolu Kırmızısı (DAK) cows. For this purpose, the locations of these specific lincRNAs and mRNAs were confirmed in quantitative trait loci. Then RT-PCR analysis was performed to identify the expression profiles of the lincRNAs and mRNAs. Lastly, correlation analysis was carried out between milk yield data from Holstein and DAK cows and expression levels of the lincRNAs and mRNAs. The findings showed that while lincRNAs and mRNAs associated with milk yield traits were upregulated in the Holstein cows exhibiting high milk yield in comparison to the DAK cows exhibiting low milk yield, lincRNA and mRNA associated with milk composition traits were downregulated in the Holstein cows with high milk yield compared to the DAK cows with low milk yield. These results suggest primary evidence for expression profiles of lincRNA and mRNA related to milk production traits in the milk-derived exosomes of Holstein and DAK cows. These lincRNAs and mRNAs, which are carried in the milk-derived exosomes, could be utilized in animal breeding programs to enhance milk yield and composition traits.

Key words: Expression, ncRNAs, traits, cattle

1. Introduction

Several nutrients, growth factors, metabolic hormones, and cytokines are found in bovine milk; it is well known that bovine milk contains important nutrients for humans [1,2]. Milk yield is one of the most important issues faced by dairy cattle farms [3]. In addition, factors such as protein and fat percentage are important determinants of milk quality [4]. Breeding studies to increase milk yield are very important for the continuity of dairy cattle farms. In recent years, the most common breeding technique used for this purpose is genomic selection, including genome-wide association studies (GWASs) [3], gene expression [4], and quantitative trait locus (QTL) [5].

Milk produced by humans and several other animal species such as cows, swine, and yaks contains different types of extracellular vesicles (EVs), such as microvesicles, exosomes, and apoptotic bodies, which play a role in several biological pathways. Moreover, EVs are related to mammary gland health. Most exosomes, which are a type of EV, are from 30 to 100 nm in size, and are released from different populations of cells into the microenvironment, under both normal and pathological events [6,7]. When proteomic analysis is performed, exosomes derived from milk can be distinguished from milk fat globule membranes by their enzymatic and transport differences [8]. Exosomes carry circulating nucleic acids, including mRNA, microRNA (miRNA), ribosomal RNA, long noncoding RNA (lncRNA), transfer RNA, and variably DNA, all of which also carry proteins. These nucleic acids, which are found in exosomes, can pass from one cell to another and affect protein production in cells [9].

With new sequencing technology, a growing number of transcripts have been identified in humans and animals. The most prominent of these transcripts are noncoding RNAs (ncRNAs). ncRNAs are miRNA, tRNA halves (tiRNAs), and Piwi-interacting RNA (piRNAs) with lengths of less than 200 bp and lncRNAs with lengths of more than 200 bp. lncRNAs can be characterized as antisense lncRNAs, intronic lncRNAs, bidirectional lncRNAs, intergenic lncRNAs (lincRNA), and sense-overlapping lncRNAs based on their locations. Recent studies have revealed the discovery of several lncRNAs in eukaryotic organisms, especially lincRNA, which play a role in chromatin modification, epigenetic regulation, genomic imprinting, and transcriptional control. Pre- and posttranslational mRNA processing has also been

* Correspondence: selcuk.ozdemir@atauni.edu.tr

identified in several animal species [10–13].

In a recent study, a large number of lincRNAs identified in the bovine mammary gland were observed in QTL. In particular, 36 lincRNAs such as TCONS_00042053, TCONS_00055411, TCONS_00068290, TCONS_00071212, TCONS_00158814, and TCONS_00135045 were found in 172 milk-related QTLs, including milk yield, milk protein content, and milk palmitic acid percentage [13]. Another study revealed genetic associations between some candidate genes and milk composition traits in Chinese Holstein cow populations. These genes are as follows: Fc fragment of the IgG receptor (*FCGR2B*), centromere-associated protein-E (*CENPE*), retinol saturase (*RETSAT*), acyl-CoA synthetase bubblegum family member 2 (*ACSBG2*), TBC1 domain family member 1 (*TBC1D1*), mitogen-activated protein kinase kinase kinase 1 (*MAP3K1*), and UDP-glucose 6-dehydrogenase (*UGDH*) [4]. A study conducted by Han et al. [14] reported that the nucleobindin 2 (*NUCB2*) gene may correlate with milk yield traits due to its expression level being significantly upregulated in early lactation or at the peak of lactation in comparison to a dry period. There are currently no data showing whether lincRNA and mRNA are carried in cow's milk-derived exosomes.

In the present study, we investigated whether lincRNAs and mRNAs found in milk-related QTL were expressed or nonexpressed in the milk-derived exosomes of Holstein and DAK cows raised in Turkey.

2. Materials and methods

2.1. Experimental animals

DAK cows are native to Turkey and raised in the Erzurum region. For the purpose of the present study, 15 multiparous, healthy, and mastitis-free Holstein and DAK cows (n = 30) were selected from two different cattle farms (Erzurum, Turkey). All cows were in their third parity and peak lactation (early peak or 90-day postpartum) milk yield was recorded regularly.

2.2. Milk sample collection

Bovine milk samples were obtained from healthy Holstein and DAK cows during peak lactation (90 days after parturition). Collected milk samples were stored at –80 °C.

2.3. Isolation of exosomes from milk

To first remove larger particles such as fat globules and cells, the milk samples were centrifuged at 5000 × *g* for 30 min at 4 °C. Afterwards, to then remove casein and other fine debris, centrifugation was applied to the samples three times at 4 °C for 1 h each at 12,000 × *g*, 35,000 × *g*, and finally 70,000 × *g*. Lastly, the samples were centrifuged at 120,000 × *g* for 4 h at 4° C using a SW41T rotor (Beckman Coulter, USA) and were maintained in a –80 ° C freezer until analysis [9].

2.4. LincRNA and mRNA in quantitative trait loci

The positions of the six lincRNAs and eight mRNAs were found on the *Bos taurus* UMD3.1 genome in accordance with the AnimalQTLdb, which is an open access database of several animal species such as cattle, chicken, horses, pigs, and sheep (http://www.animalgenome.org/QTLdb/) [15].

2.5. Total RNA extraction and cDNA processes

Total RNA was isolated from milk-derived exosomes using TRIzol (Invitrogen, Cat: 15596026, USA) according to the manufacturer's instructions. After total RNA isolation, the concentration of RNA was determined with a NanoDrop (Epoch Microplate Spectrophotometer, USA). Later, the quality of total RNA samples was evaluated with gel electrophoresis (Figure S1). cDNA synthesis was done with QuantiTect reverse transcription (Qiagen, Cat: 330411 Germany) [16,17].

2.6. qPCR

qRT-PCR measures the transcript levels of TCONS_00042053, TCONS_00055411, TCONS_00068290, TCONS_00071212, TCONS_00158814, TCONS_00135045, *FCGR2B*, *CENPE*, *RETSAT*, *ACSBG2*, *TBC1D1*, *MAP3K1*, *UGDH*, and *NUCB2* in the milk-derived exosomes using a ROTOR-GENE Q 5plex HRM Real-Time PCR Detection System (Qiagen, Germany). GAPDH and beta-actin were used as internal control genes. Specific primers were prepared with the primer design program Primer 5.0. All primer sequences and reaction conditions are shown in supplementary files 1 and 2. 2x QuantiTect SYBR Green PCR Master Mix (Qiagen, Cat: 330500, Germany) was used for qPCR. The qPCR products were evaluated with agarose gel electrophoresis and melting curve analysis [16,17], and the expression fold changes were determined in accordance with the $2^{-\Delta\Delta CT}$ method [18].

2.7. Protein–protein interaction (PPI) analysis

PPI analysis was performed for *FCGR2B*, *CENPE*, *RETSAT*, *ACSBG2*, *TBC1D1*, *MAP3K1*, *UGDH*, and *NUCB2* to identify interactions using the STRING database.

2.8. Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the statistical differences of TCONS_00042053, TCONS_00055411, TCONS_00068290, TCONS_00071212, TCONS_00158814, TCONS_00135045, *FCGR2B*, *CENPE*, *RETSAT*, *ACSBG2*, *TBC1D1*, *MAP3K1*, *UGDH*, and *NUCB2*. GraphPad Prism software (Version 7.0, California, USA) was used to examine the expression fold changes. For correlation analysis, the CORR procedure was used in the package program SAS 9.4. The given coefficients are Pearson correlation coefficients.

3. Results

3.1. Functional prediction of lincRNA and mRNA

The locations of six lincRNAs and eight mRNAs were compared using AnimalQTLdb in order to predict functions related to milk yield traits. These lincRNAs and mRNAs were clustered in several QTL regions, including milk yield, protein yield and protein percentage, fat yield and protein percentage, milk palmitic acid percentage, body weight, and somatic cell count (Figure 1). These results suggest that TCONS_00042053, TCONS_00055411, TCONS_00068290, TCONS_00071212, TCONS_00158814, TCONS_00135045, *FCGR2B*, *CENPE*, *RETSAT*, *ACSBG2*, *TBC1D1*, *MAP3K1*, *UGDH*, and *NUCB2* play a role in milk secretion.

3.2. Expression profiles of lincRNA between Holstein and DAK milk

We assessed the relative expression levels of TCONS_00042053, TCONS_00055411, TCONS_00068290, TCONS_00071212, TCONS_00158814, and TCONS_00135045 in the milk-derived exosomes of Holstein and DAK cows. LincRNAs related to milk yield, including TCONS_00042053, TCONS_00055411, TCONS_00068290, and TCONS_00071212, were upregulated in the exosomes of Holstein milk compared to DAK milk. However, lincRNAs related to protein yield and protein percentage, and milk palmitic acid percentage, including TCONS_00068290 and TCONS_00071212 were downregulated in the exosomes of Holstein milk compared to DAK milk (Figures 2A–2F) ($P < 0.05$ and $P < 0.01$). The amplification peaks of lincRNAs are shown in Figure S2.

3.3. Expression profiles of mRNA between Holstein and DAK milk

The mRNA transcript levels of *FCGR2B*, *CENPE*, *RETSAT*, *ACSBG2*, *TBC1D1*, *MAP3K1*, *UGDH*, and *NUCB2* were evaluated in the milk-derived exosomes of the Holstein and DAK cows. mRNA-related milk production traits were upregulated in the exosomes of Holstein milk compared to DAK milk. Alternatively, mRNA-related protein yield and protein percentage, and fat yield and protein percentage were downregulated in the exosomes of Holstein milk compared to DAK milk (Figures 3A–3H) ($P < 0.01$). The amplification peaks of mRNA are shown in Figure S3.

3.4. Pearson correlation analysis

In the correlation analysis between the milk yield data of the Holstein cows and the expression data of the two lincRNAs (TCONS_00158814, and TCONS_00135045) associated with fat yield and protein percentage, milk palmitic acid percentage showed a negative correlation, whereas other lincRNAs related to milk yield showed a positive correlation. Furthermore, there was a negative correlation between high milk yield and the expression level of mRNAs (*TBC1D1*, *MAP3K1*) associated with milk composition traits, and a positive correlation between high milk yield and the expression level of mRNAs associated with milk production traits in the Holstein cows (Table 1). In the DAK cows, two significant negative correlations were found between milk yield and TCONS_00042053/*FCGR2B*. However, a significant correlation between milk yield data of the DAK cows and the expression data of other lincRNA/mRNA was not found (Table 2).

3.5. Protein–protein interactions

We observed that *CENPE* and *UGDH* were coexpressed, as were *RETSAT* and *ACSBG2*. In addition, we demonstrated interactions between *FCGR2B*, *CENPE*, *RETSAT*, *ACSBG2*, *TBC1D1*, and *UGDH*; however, there were no interactions of *MAP3K1* and *NUCB2* with other mRNAs (Figure 4).

4. Discussion

Milk yield and content are economically important for dairy cattle farms and are affected by a large number of environmental factors and genes [19–22]. Recent studies

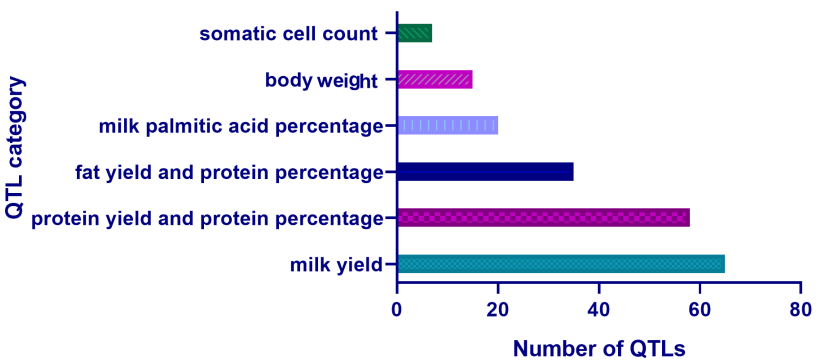

Figure 1. Number of QTLs associated with lincRNAs and mRNAs. Milk-related QTL was top among all QTLs, milk yield, protein yield and protein percentage, fat yield and protein percentage, milk palmitic acid percentage, body weight, and somatic cell count.

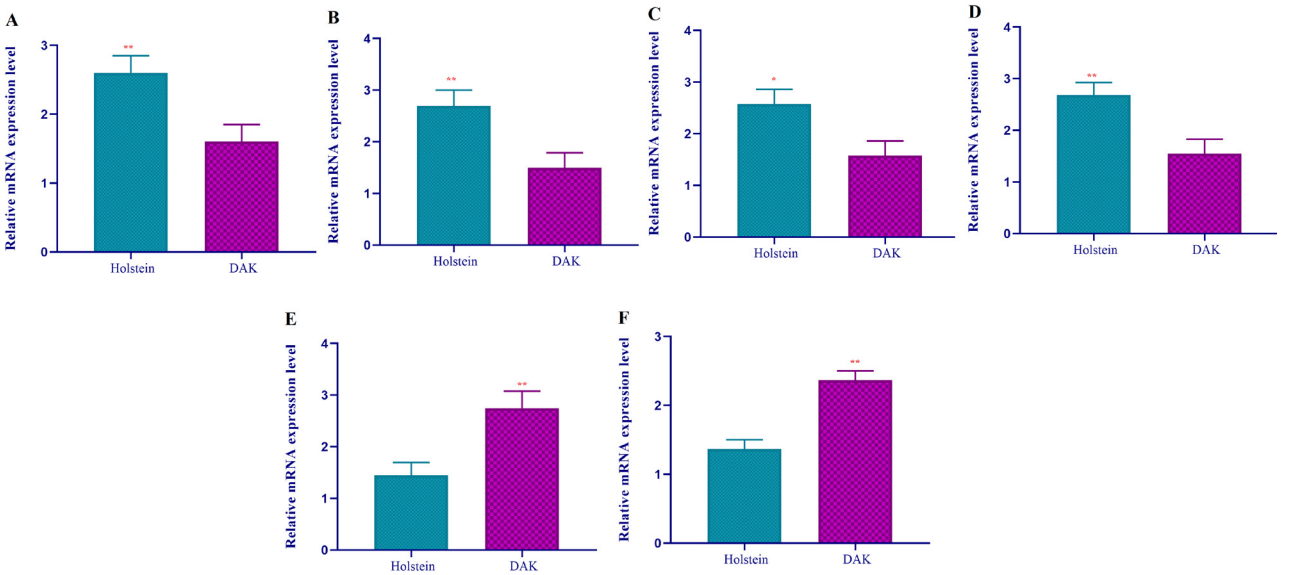

Figure 2. mRNA transcript levels of the lincRNAs in the milk-derived exosomes of Holstein and DAK cows. Values represent the mean ± SD of 3 independent samples. Statistical significance (*P < 0.05, **P < 0.01) was analyzed by one-way ANOVA. A–F represent the relative expression levels of TCONS_00042053, TCONS_00055411, TCONS_00068290, TCONS_00071212, TCONS_00158814, and TCONS_00135045, respectively.

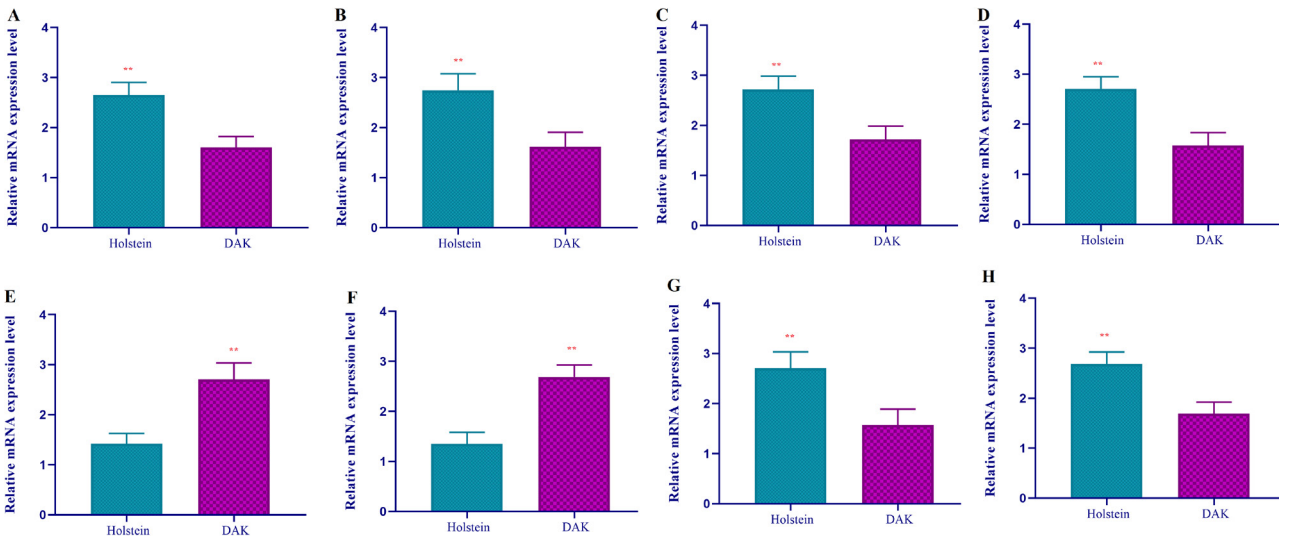

Figure 3. mRNA transcript levels of the mRNAs in the milk-derived exosomes of Holstein and DAK cows. Values represent the mean ± SD of 3 independent samples. The error bars show the standard deviation. Statistical significance (*P < 0.05, **P < 0.01) was analyzed by one-way ANOVA. A–G represent the relative expression levels of *FCGR2B*, *CENPE*, *RETSAT*, *ACSBG2*, *TBC1D1*, *MAP3K1*, *UGDH*, and *NUCB2*, respectively.

have revealed several genes and mutations related to milk yield and composition traits in cows. The most important studies on milk yield are QTL and GWASs, which have been performed to detect QTL regions, genes, ncRNAs, and mutations that impact milk yield traits in cows [13,23–27]. A commonly accessed genetic database for detecting numerous QTL and genetic association in animals is: http://www.animalgenome.org/cgi-bin/QTLdb/index [4,28,29].

A previous study reported a connection between 36 lincRNAs and 172 milk-related QTLs detected in bovine mammary gland tissue. For example, TCONS_00042053, TCONS_00055411, TCONS_00068290, TCONS_00071212, TCONS_00158814, and

Table 1. Pearson correlation results between Holstein milk yield and the expression fold changes in lincRNAs/mRNAs.

		Peak_lactation__90d__Milk_yield_
TCONS_00042053__Fold_change_	r P	0.90952** <0.0001
TCONS_00055411__Fold_change_	r P	0.86238** <0.0001
TCONS_00068290__Fold_change_	r P	0.94945** <0.0001
TCONS_00071212__Fold_change_	r P	0.95921** <0.0001
TCONS_00158814__Fold_change_	r P	**–0.88272**** **<0.0001**
TCONS_00135045__Fold_change_	r P	**–0.89106**** **<0.0001**
FCGR2B__Fold_change_	r P	0.73418** 0.0018
CENPE___Fold_change_	r P	0.87011** <.0001
MAP3K1__Fold_change_	r P	0.8533** <0.0001
RETSAT__Fold_change_	r P	0.92793** <0.0001
ACSBG2__Fold_change_	r P	**–0.76336**** **0.0009**
TBC1D1__Fold_change_	r P	**–0.77356**** **0.0007**
UGDH___Fold_change_	r P	0.87494** <0.0001
NUCB2__Fold_change_	r P	0.7506** 0.0013

Table 2. Pearson correlation results between DAK milk yield and the expression fold changes in lincRNAs/mRNAs lincRNAs/mRNAs.

		Peak_lactation__90d__Milk_yield_
TCONS_00042053__Fold_change_	r P	**–0.60395*** **0.0171**
TCONS_00055411__Fold_change_	r P	0.33931 NS
TCONS_00068290__Fold_change_	r P	–0.35314 NS
TCONS_00071212__Fold_change_	r P	0.09169 NS
TCONS_00158814__Fold_change_	r P	0.25755 NS
TCONS_00135045__Fold_change_	r P	**–0.56336*** **0.0288**
FCGR2B__Fold_change_	r P	–0.43653 NS
CENPE___Fold_change_	r P	–0.08519 NS
MAP3K1__Fold_change_	r P	–0.28993 NS
RETSAT__Fold_change_	r P	–0.00711 NS
ACSBG2__Fold_change_	r P	–0.48169 NS
TBC1D1__Fold_change_	r P	–0.27464 NS
UGDH___Fold_change_	r P	0.0078 NS
NUCB2__Fold_change_	r P	–0.27464 NS

TCONS_00135045 were all detected. In addition, milk-related QTL regions related to these lincRNAs are milk yield, milk protein percentage, and milk palmitic acid percentage [13]. However, data on expression profiles of TCONS_00042053, TCONS_00055411, TCONS_00068290, TCONS_00071212, TCONS_00158814, and TCONS_00135045 in the milk-derived exosomes of Holstein and DAK cows have not yet been detected. In the present study, we confirmed that these lincRNAs were related to milk yield, protein yield and protein percentage, fat yield and protein percentage, milk palmitic acid percentage, body weight, and somatic cell count using AnimalQTLdb. Moreover, we identified the expression patterns of these lincRNAs using RT-PCR to examine the milk-derived exosomes of Holstein and DAK cows. Our findings reveal that lincRNAs associated with milk yield, such as TCONS_00042053, TCONS_00055411, TCONS_00068290, and TCONS_00071212, were upregulated in Holstein cows with high milk yield compared to DAK cows with low milk yield. However, lincRNAs that are related to fat yield and protein percentage or milk palmitic acid percentage such as TCONS_00158814 and TCONS_00135045 were downregulated in Holstein cows with high milk yield compared to DAK cows with low milk yield. Meanwhile, when the correlation analysis between the milk yield data of these cows and the expression data of the two lincRNAs associated with fat yield and protein percentage or milk palmitic acid percentage were found to show a negative correlation, other lincRNAs related to milk yield were found to show a positive correlation. These

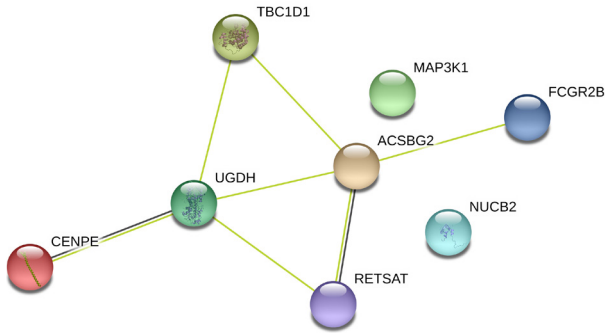

Figure 4. Protein–protein interaction analysis of significantly differentially expressed proteins. Icon in each circle indicates the three-dimensional structure of proteins. No icon indicates that there was no information on protein structure in the STRING database. Protein–protein interactions results for *FCGR2B*, *CENPE*, *RETSAT*, *ACSBG2*, *TBC1D1*, *MAP3K1*, *UGDH*, and *NUCB2*.

results revealed that the lincRNAs that are associated with milk production traits and milk composition traits were carried in the milk-derived exosomes of cows. This was also reported in a previous study in which 12 differentially expressed lncRNAs potentially played an important role in bovine lactation [27]. In another study, 12 lncRNAs found in milk exosomes during different stages of lactation (colostrum at 2 days, 30 days, 150 days, and 270 days) showed variations across the stages [30]. Both our findings and previous studies' results showed that lincRNA and lncRNA potentially played a role in milk secretion and milk composition in cows.

Previous studies have shown that *FCGR2B*, *CENPE*, *RETSAT*, *ACSBG2*, *TBC1D1*, *MAP3K1*, *UGDH*, and *NUCB2* genes were associated with milk composition traits and milk production traits in dairy cows [4,14]. However, there is no study on the expression profiles of these genes in the milk-derived exosomes of Holstein and DAK cows. In the present study, we observed that these mRNAs were related to milk composition traits and milk production traits using AnimalQTLdb similar to lincRNAs. We also revealed the expression profiles of these mRNAs in the milk-derived exosomes of Holstein and DAK cows. According to our results, while the transcription levels of mRNAs related to milk production traits, including *FCGR2B*, *CENPE*, *RETSAT*, *ACSBG2*, *UGDH*, and *NUCB2*, were increased in Holstein cows with high milk yield in comparison to DAK cows with low milk yield, the transcriptional levels of mRNAs related to milk composition traits were decreased in the Holstein cows with high milk yield compared to the DAK cows with low milk yield. In addition to these results, correlation analysis showed that there is a negative correlation between high milk yield and the expression level of mRNAs associated with milk composition traits, and a positive correlation between high milk yield and the expression level of mRNAs associated with milk production traits. These results were compatible with our lincRNAs and previous studies' findings.

In the present study, the numbers of QTLs associated with lincRNAs and mRNAs (milk-related QTL was top among all QTLs, milk yield, protein yield and protein percentage, fat yield and protein percentage, milk palmitic acid percentage, body weight, and somatic cell count) were determined. The difference in expression between high and low milk yield cows could be attributed to the fact that candidate lincRNA and mRNA gene targets are more correlated with milk yield.

Genomic selection is essential for productive dairy cattle breeding, and the development of sequencing technology provides evaluations of nucleic acid marker technology and genomics, which accelerate the rate of genomic selection for economic traits [31]. The genomic loci associated with milk yield and milk composition traits could be used in the field of genomic selection to increase milk yield in dairy cattle [32–35]. In the present study, we identified expression profiles of lincRNAs and mRNAs associated with milk yield, protein yield and protein percentage, fat yield and protein percentage, and milk palmitic acid percentage in the milk-derived exosomes of Holstein and DAK cows. These lincRNAs and mRNAs that show significant genetic effects on milk traits could be used to increase the effectiveness of selection for milk production in dairy cattle.

5. Conclusions

This research reveals the expression profiles of lincRNAs and mRNAs, including TCONS_00042053, TCONS_00055411, TCONS_00068290, TCONS_00071212, TCONS_00158814, TCONS_00135045, *FCGR2B*, *CENPE*, *RETSAT*, *ACSBG2*, *TBC1D1*, *MAP3K1*, *UGDH*, and *NUCB2* in the milk-derived exosomes of Holstein and DAK cows. Our results show that these lincRNAs and mRNAs, which are carried in milk-derived exosomes, could be used in animal breeding programs to enhance milk yield and composition traits.

Acknowledgment

We thank the Eastern Anatolia Advanced Technology Application and Research Center (DAYTAM) of Atatürk University for its support.

Conflict of Interest

The authors declare that they have no competing interests.

Financial Support

This study did not receive any specific grand from funding agencies in the public, commercial or not for profit sectors.

References

1. van Hooijdonk AC, Kussendrager KD, Steijns JM. *In vivo* antimicrobial and antiviral activity of components in bovine milk and colostrum involved in non-specific defence. The British Journal of Nutrition 2000; 84 (1): 127-134. doi: 10.1017/s000711450000235x
2. Egger-Danner C, Cole JB, Pryce JE, Gengler N, Heringstad B et al. Invited review: overview of new traits and phenotyping strategies in dairy cattle with a focus on functional traits. Animal: An International Journal of Animal Bioscience 2015; 9 (2): 191-207. doi: 10.1017/s1751731114002614
3. Oliveira HR, Cant JP, Brito LF, Feitosa FLB, Chud TCS et al. Genome-wide association for milk production traits and somatic cell score in different lactation stages of Ayrshire, Holstein, and Jersey dairy cattle. Journal of Dairy Science 2019; 102 (9): 8159-8174. doi: 10.3168/jds.2019-16451
4. Jiang J, Liu L, Gao Y, Shi L, Li Y et al. Determination of genetic associations between indels in 11 candidate genes and milk composition traits in Chinese Holstein population. BMC Genetics 2019; 20 (1): 48. doi: 10.1186/s12863-019-0751-y
5. Seno LO, Guidolin DGF, Aspilcueta-Borquis RR, Nascimento GBD, Silva T et al. Genomic selection in dairy cattle simulated populations. Journal of Dairy Research 2018; 85 (2): 125-132. doi: 10.1017/s0022029918000304
6. Thery C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Current Protocols in Cell Biology 2006; Chapter 3: Unit 3.22. doi: 10.1002/0471143030.cb0322s30
7. Ono S, Lam S, Nagahara M, Hoon DS. Circulating microRNA biomarkers as liquid biopsy for cancer patients: pros and cons of current assays. Journal of Clinical Medicine 2015; 4 (10): 1890-1907. doi: 10.3390/jcm4101890
8. Reinhardt TA, Lippolis JD, Nonnecke BJ, Sacco RE. Bovine milk exosome proteome. Journal of Proteomics 2012; 75 (5): 1486-1492. doi: 10.1016/j.jprot.2011.11.017
9. Hata T, Murakami K, Nakatani H, Yamamoto Y, Matsuda T et al. Isolation of bovine milk-derived microvesicles carrying mRNAs and microRNAs. Biochemical and Biophysical Research Communications 2010; 396 (2): 528-533. doi: 10.1016/j.bbrc.2010.04.135
10. Ulitsky I, Bartel DP. lincRNAs: genomics, evolution, and mechanisms. Cell 2013; 154 (1): 26-46. doi: 10.1016/j.cell.2013.06.020
11. Ulitsky I. Evolution to the rescue: using comparative genomics to understand long non-coding RNAs. Nature Reviews Genetics 2016; 17 (10): 601-614. doi: 10.1038/nrg.2016.85
12. Geisler S, Coller J. RNA in unexpected places: long non-coding RNA functions in diverse cellular contexts. Nature Reviews Molecular Cell Biology 2013; 14 (11): 699-712. doi: 10.1038/nrm3679
13. Tong C, Chen Q, Zhao L, Ma J, Ibeagha-Awemu EM et al. Identification and characterization of long intergenic noncoding RNAs in bovine mammary glands. BMC Genomics 2017; 18 (1): 468. doi: 10.1186/s12864-017-3858-4
14. Han B, Yuan Y, Li Y, Liu L, Sun D. Single nucleotide polymorphisms of NUCB2 and their genetic associations with milk production traits in dairy cows. Genes 2019; 10 (6): 449. doi: 10.3390/genes10060449
15. Hu ZL, Park CA, Reecy JM. Developmental progress and current status of the Animal QTLdb. Nucleic Acids Research 2016; 44 (1): 827-833. doi: 10.1093/nar/gkv1233
16. Ozdemir S, Comakli S. Investigation of the interaction between bta-miR-222 and the estrogen receptor alpha gene in the bovine ovarium. Reproductive Biology 2018; 18 (3): 259-266. doi: 10.1016/j.repbio.2018.06.006
17. Arslan H, Ozdemir S, Altun S. Cypermethrin toxication leads to histopathological lesions and induces inflammation and apoptosis in common carp (*Cyprinus carpio* L.). Chemosphere 2017; 180: 491-499. doi: 10.1016/j.chemosphere.2017.04.057
18. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25 (4): 402-408. doi: 10.1006/meth.2001.1262
19. Blott S, Kim JJ, Moisio S, Schmidt-Kuntzel A, Cornet A et al. Molecular dissection of a quantitative trait locus: a phenylalanine-to-tyrosine substitution in the transmembrane domain of the bovine growth hormone receptor is associated with a major effect on milk yield and composition. Genetics 2003; 163 (1): 253-266.
20. Grisart B, Farnir F, Karim L, Cambisano N, Kim JJ et al. Genetic and functional confirmation of the causality of the DGAT1 K232A quantitative trait nucleotide in affecting milk yield and composition. Proceedings of the National Academy of Sciences of the United States of America 2004; 101 (8): 2398-2403. doi: 10.1073/pnas.0308518100
21. Cohen-Zinder M, Seroussi E, Larkin DM, Loor JJ, Everts-van der Wind A et al. Identification of a missense mutation in the bovine ABCG2 gene with a major effect on the QTL on chromosome 6 affecting milk yield and composition in Holstein cattle. Genome Research 2005; 15 (7): 936-944. doi: 10.1101/gr.3806705
22. Yang SH, Bi XJ, Xie Y, Li C, Zhang SL et al. Validation of PDE9A gene identified in GWAS showing strong association with milk production traits in Chinese Holstein. International Journal of Molecular Sciences 2015; 16 (11): 26530-26542. doi: 10.3390/ijms161125976
23. Georges M, Nielsen D, Mackinnon M, Mishra A, Okimoto R et al. Mapping quantitative trait loci controlling milk production in dairy cattle by exploiting progeny testing. Genetics 1995; 139 (2): 907-920.
24. Andersson L. Genome-wide association analysis in domestic animals: a powerful approach for genetic dissection of trait loci. Genetics 2009; 136 (2): 341-349. doi: 10.1007/s10709-008-9312-4

25. Schennink A, Bovenhuis H, Leon-Kloosterziel KM, van Arendonk JA, Visker MH. Effect of polymorphisms in the FASN, OLR1, PPARGC1A, PRL and STAT5A genes on bovine milk-fat composition. Animal Genetics 2009; 40 (6): 909-916. doi: 10.1111/j.1365-2052.2009.01940.x

26. Li C, Sun D, Zhang S, Wang S, Wu X et al. Genome wide association study identifies 20 novel promising genes associated with milk fatty acid traits in Chinese Holstein. PloS One 2014; 9 (5): e96186. doi: 10.1371/journal.pone.0096186

27. Zheng X, Ning C, Zhao P, Feng W, Jin Y et al. Integrated analysis of long noncoding RNA and mRNA expression profiles reveals the potential role of long noncoding RNA in different bovine lactation stages. Journal of Dairy Science 2018; 101 (12): 11061-11073. doi: 10.3168/jds.2018-14900

28. Kunieda M, Tsuji T, Abbasi AR, Khalaj M, Ikeda M et al. An insertion mutation of the bovine Fii gene is responsible for factor XI deficiency in Japanese black cattle. Mammalian Genome: Official Journal of the International Mammalian Genome Society 2005; 16 (5): 383-389. doi: 10.1007/s00335-004-2462-5

29. Kerje S, Sharma P, Gunnarsson U, Kim H, Bagchi S et al. The Dominant white, Dun and Smoky color variants in chicken are associated with insertion/deletion polymorphisms in the PMEL17 gene. Genetics 2004; 168 (3): 1507-1518. doi: 10.1534/genetics.104.027995

30. Zeng B, Chen T, Xie MY, Luo JY et al. Exploration of long noncoding RNA in bovine milk exosomes and their stability during digestion in vitro. Journal of Dairy Science 2019; 102 (8): 6726-6737. doi: 10.3168/jds.2019-16257

31. Wiggans GR, Cole JB, Hubbard SM, Sonstegard TS. Genomic selection in dairy cattle: the USDA experience. Annual Review of Animal Biosciences 2017; 5 (1): 309-327. doi: 10.1146/annurev-animal-021815-111422

32. Bouwman AC, Bovenhuis H, Visker MH, van Arendonk JA. Genome-wide association of milk fatty acids in Dutch dairy cattle. BMC Genetics 2017; 12: 43. doi: 10.1186/1471-2156-12-43

33. Matsumoto H, Sasaki K, Bessho T, Kobayashi E, Abe T et al. The SNPs in the ACACA gene are effective on fatty acid composition in Holstein milk. Molecular Biology Reports 2012; 39 (9): 8637-8644. doi: 10.1007/s11033-012-1718-5

34. Bhattarai D, Chen X, Ur Rehman Z, Hao X, Ullah F et al. Association of MAP4K4 gene single nucleotide polymorphism with mastitis and milk traits in Chinese Holstein cattle. Journal of Dairy Research 2017; 84 (1): 76-79. doi: 10.1017/s0022029916000832

35. Han B, Liang W, Liu L, Li Y, Sun D. Genetic association of the ACACB gene with milk yield and composition traits in dairy cattle. Animal Genetics 2018; 49 (3): 169-177. doi: 10.1111/age.12651

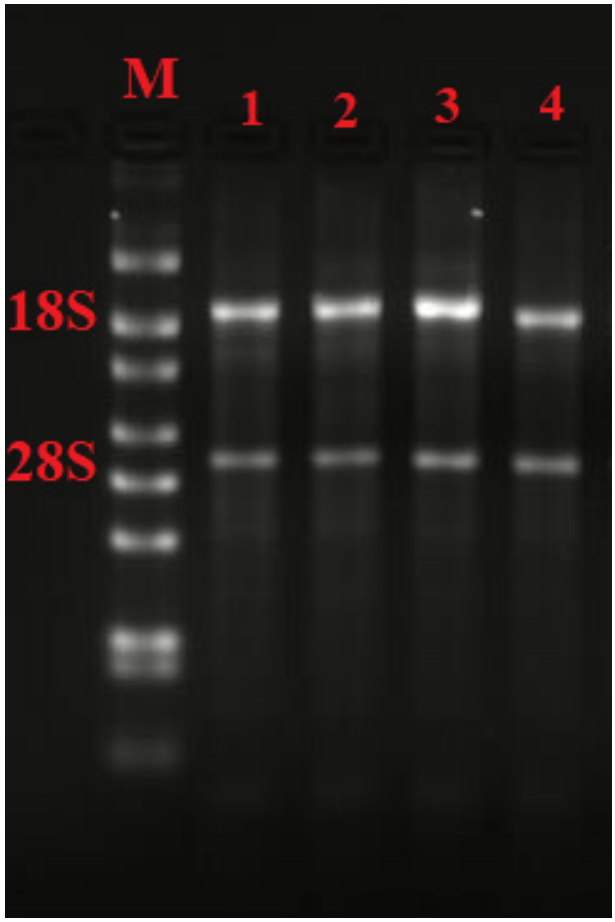

Figure S1. Gel electrophoresis results of total RNA samples isolated from milk-derived exosomes. M: RNA ladder. Panels 1 and 2: Total RNA samples from milk-derived exosomes of Holstein cows. Panels 3 and 4: Total RNA samples from milk-derived exosomes of DAK cows.

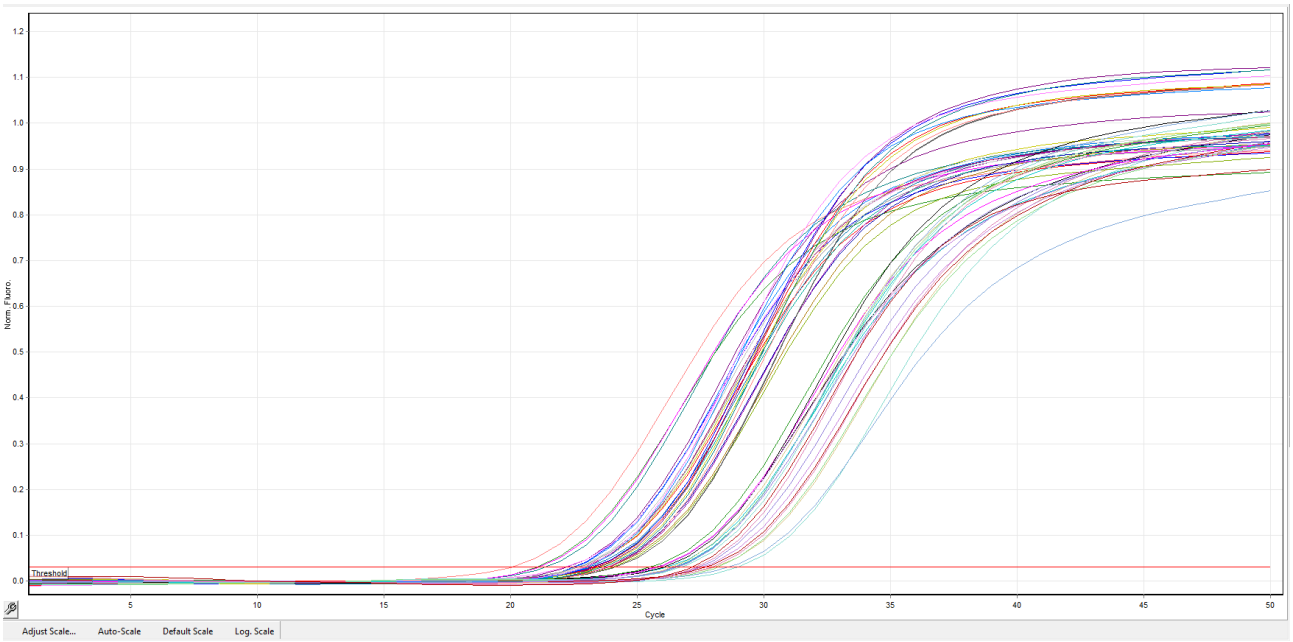

Figure S2. The fluorescence signal (RFU) vs. Cq [threshold time (min)] amplification curve graph was plotted automatically by the ROTOR-GENE Q 5plex HRM Real-Time PCR Detection System (Qiagen, Germany). Amplification peaks for lincRNAs obtained by RT-PCR.

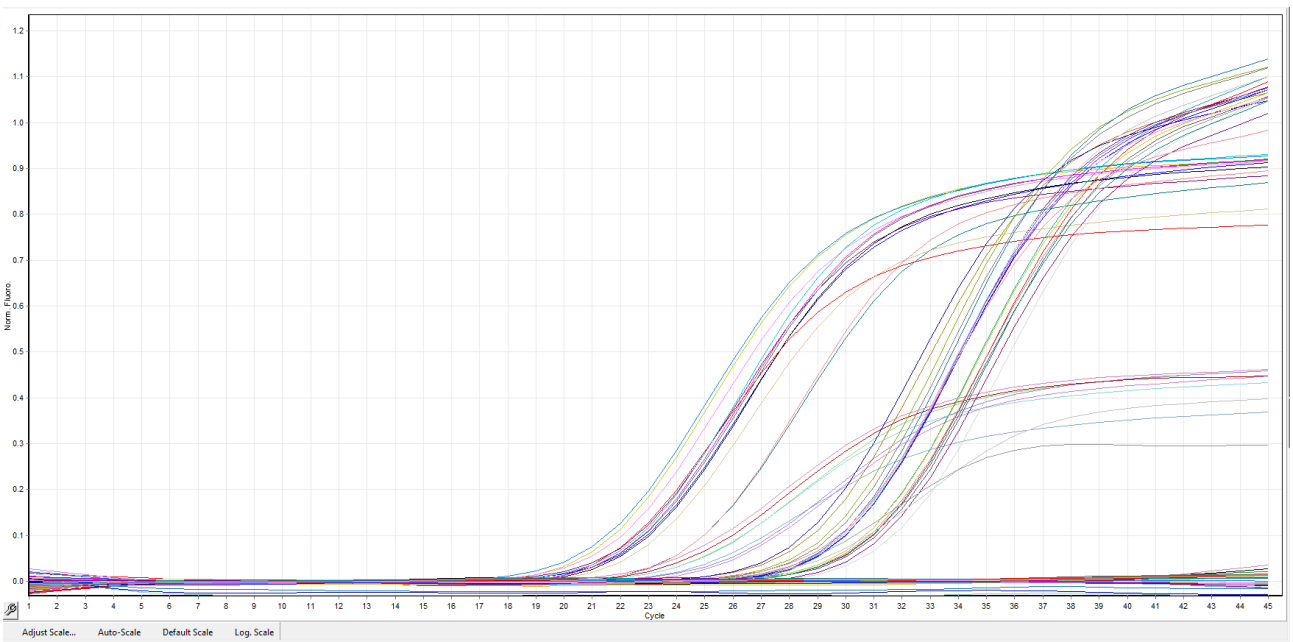

Figure S3. The fluorescence signal (RFU) vs. Cq [threshold time (min)] amplification curve graph was plotted automatically by the ROTOR-GENE Q 5plex HRM Real-Time PCR Detection System (Qiagen, Germany). Amplification peaks for mRNAs obtained by RT-PCR.

Supplementary file 1. Primers of lincRNAs used in qPCR.

Transcript name	Transcript type	Primer sequences	Product length (bp)	QTL region	Chromosome	Start–End	References
TCONS_00042053	lincRNA	F: AGGCTCTTAGGGCAGAAGGA R: TGTCTGTTTGCACAAGATTGTCT	376	Milk Yield	15	43615909–45451865	Tong et al., 2017
TCONS_00055411	lincRNA	F: ACGGAGAGGGAGACTTCTGA R: AGAAGATGCGACCTTGACCC	293	Milk Yield	17	72084037–73565104	Tong et al., 2017
TCONS_00068290	lincRNA	F: ACACTCCCACGCCTGGATCT R: CCAAGGAGGTGCAGACCA	266	Milk Yield	18	64046302–64709974	Tong et al., 2017
TCONS_00071212	lincRNA	F: ACTGCTGCTTGGCTCAGTTA R: AGGTCTAGATGGATTTCACGATGG	299	Milk Yield	19	61806709–62390766	Tong et al., 2017
TCONS_00158814	lincRNA	F: TCACAGCAGCTGGGTATCAC R: CATCTTGTGGCTGTGTCGTG	283	Milk protein content	6	18046673–47449338	Tong et al., 2017
TCONS_00135045	lincRNA	F: TTCCAGAAGGTCCTGAAGTCG R: ACTGTAAGATGGACACCCTGCG	201	Milk palmitic acid percentage	3	118167062–121366345	Tong et al., 2017

Supplementary file 2. Primers of mRNAs used in qPCR.

Transcript name	Transcript type	Primer sequences	Product length (bp)	QTL region	Accession number	References
FCGR2B	mRNA	F: CATAACGGGAGCTCCATCCA R: TGAGACCGGCTTGGACAGT	400	Milk yield, protein yield, and protein percentage	XM_005203449.4	Jiang et al., 2016
CENPE	mRNA	F: GCAACAAAGCTACTAAGTCAGGAAA R: ACTTCTGCATGCTTAACTAAATTCT	245	Milk yield, protein yield, and protein percentage	XM_010805938.3	Jiang et al., 2016
MAP3K1	mRNA	F: CCATTCAGTTGCAAAGCGGT R: TGGTTTTACCACCACAGGGC	333	Milk yield, protein yield, and protein percentage	XM_005221498.4	Jiang et al., 2016
RETSAT	mRNA	F: GGACTATCTAACTGAGCGGAGC R: ACGGAGAAAGCTTGCTTGAGA	299	Milk yield, protein yield, and protein percentage	XM_027555556.1	Jiang et al., 2016
ACSBG2	mRNA	F: CCGCATTTTTATCAGCGGGG R: ACAAGGCGTGACTTTGATGC	379	Fat yield and protein percentage	XM_024994963.1	Jiang et al., 2016
TBC1D1	mRNA	F: CTGGGTGGCCAAGGTGC R: GCCTGCATCCTGAAACTCCT	349	Fat yield and protein percentage	XM_024993033.1	Jiang et al., 2016
UGDH	mRNA	F: GGAAGTGGCCAGGCCTAAA R: TGCCAAGAACTCAGGGTTGG	338	Milk yield	XM_024992987.1	Jiang et al., 2016
NUCB2	mRNA	F: TAGAACTACAGTGCAGAGCCG R: GATGGTCCTCCACTTCATCTTC	292	Milk production traits	XM_005215930.3	Han et al., 2019